Linking JNK signaling to NF-κB: a key to survival

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Summary

In addition to marshalling immune and inflammatory responses, transcription factors of the NF-κB family control cell survival. This control is crucial to a wide range of biological processes, including B and T lymphopoiesis, adaptive immunity, oncogenesis and cancer chemoresistance. During an inflammatory response, NF-κB activation antagonizes apoptosis induced by tumor necrosis factor (TNF)-α, a protective activity that involves suppression of the Jun N-terminal kinase (JNK) cascade. This suppression can involve upregulation of the Gadd45-family member Gadd45β/Myd118, which associates with the JNK kinase MKK7/JNKK2 and blocks its catalytic activity. Upregulation of XIAP, A20 and blockers of reactive oxygen species (ROS) appear to be important additional means by which NF-κB blunts JNK signaling. These recent findings might open up entirely new avenues for therapeutic intervention in chronic inflammatory diseases and certain cancers; indeed, the Gadd45β-MKK7 interaction might be a key target for such intervention.

Key words: NF-κB, JNK, Gadd45β, TNF-α, Apoptosis

Introduction

In multicellular organisms, cells are constantly faced with the choice of whether to live or die. The decision requires integration of a complex network of intracellular and extracellular signals, and making the right decision is essential for survival of these organisms. Programmed cell death (PCD) is crucial to tissue homeostasis, organ development and the elimination of defective or 'dangerous' cells, such as cancerous and virus-infected cells (Danial and Korsmeyer, 2004; Rathmell and Thompson, 2002). Underscoring the importance of this process, numerous diseases arise from defects in the pathways controlling PCD. For instance, defective and excessive cell death respectively contribute to cancer and neurodegenerative disorders such as Alzheimer's disease (Danial and Korsmeyer, 2004; Rathmell and Thompson, 2002). Ultimately, the balance between life and death might depend on the ability of the cell to sustain activation of transcription factors of the NF-κB family.

Among the various functions of members of the NF-κB family, the control of PCD has arguably received the most attention in recent years. This activity is crucial for normal organismal physiology and is implicated in several pathological conditions, including cancer and chronic inflammation (Gerondakis and Strasser, 2003; Karin and Lin, 2002; Kucharczac et al., 2003; Orlowski and Baldwin, 2002). Indeed, inhibitors of NF-κB are becoming drugs of choice in the treatment of an increasing number of illnesses (Amit and Ben-Neriah, 2003; Karin et al., 2004; Tak and Firestein, 2001). However, as NF-κB has numerous functions, particularly in immunity, there is a need for new compounds that selectively target the pro-survival activity of NF-κB and thereby minimize deleterious effects on the immune system. Remarkably, this goal now appears realistic, because it is becoming increasingly clear that – although integrated – the functions of NF-κB in immunity and PCD are executed through distinct subsets of target genes.

The functions and regulation of NF-κB signaling have been the subjects of excellent recent reviews (Chen and Greene, 2004; Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000; Kucharczak et al., 2003; Li and Verma, 2002; Silvermann and Maniatis, 2001). Here, we focus instead on recent discoveries that have revealed how NF-κB, stimulated by tumor necrosis factor (TNF)-α, controls PCD by engaging in a crosstalk with the JNK MAP kinase cascade – a signaling pathway that is known to promote apoptosis. We go on to discuss the relevance of this crosstalk to inhibition of PCD in animal physiology and disease.

The NF-κB family

In vertebrates and invertebrates, NF-κB-family transcription factors are master coordinators of immune and inflammatory responses (reviewed by Chen and Greene, 2004; Karin and Ben-Neriah, 2000; Li and Verma, 2002; Silvermann and Maniatis, 2001). They also promote cell survival. In mammals, the family consists of Rel (c-Rel), RelA (p65), RelB, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). Each polypeptide has a Rel-homology domain (RHD), which mediates both DNA binding and dimerization. Usually, ubiquitous NF-κB dimers are sequestered in the cytoplasm by inhibitory IκB proteins (IκBα, IκBβ and IκBε) and are activated rapidly by stimuli that induce the sequential phosphorylation and proteolysis of IκBs – a process that depends on the IκB kinase (IKK) complex and the ubiquitin/proteasome pathway (Fig. 1). Upon removal of the inhibitors, NF-κB dimers enter nuclei to induce expression of coordinate sets of target genes that regulate innate and adaptive immunity, inflammation, cell growth and cell survival. A non-canonical pathway for NF-κB activation, involving proteolytic processing of p52/p100, has also been recently described

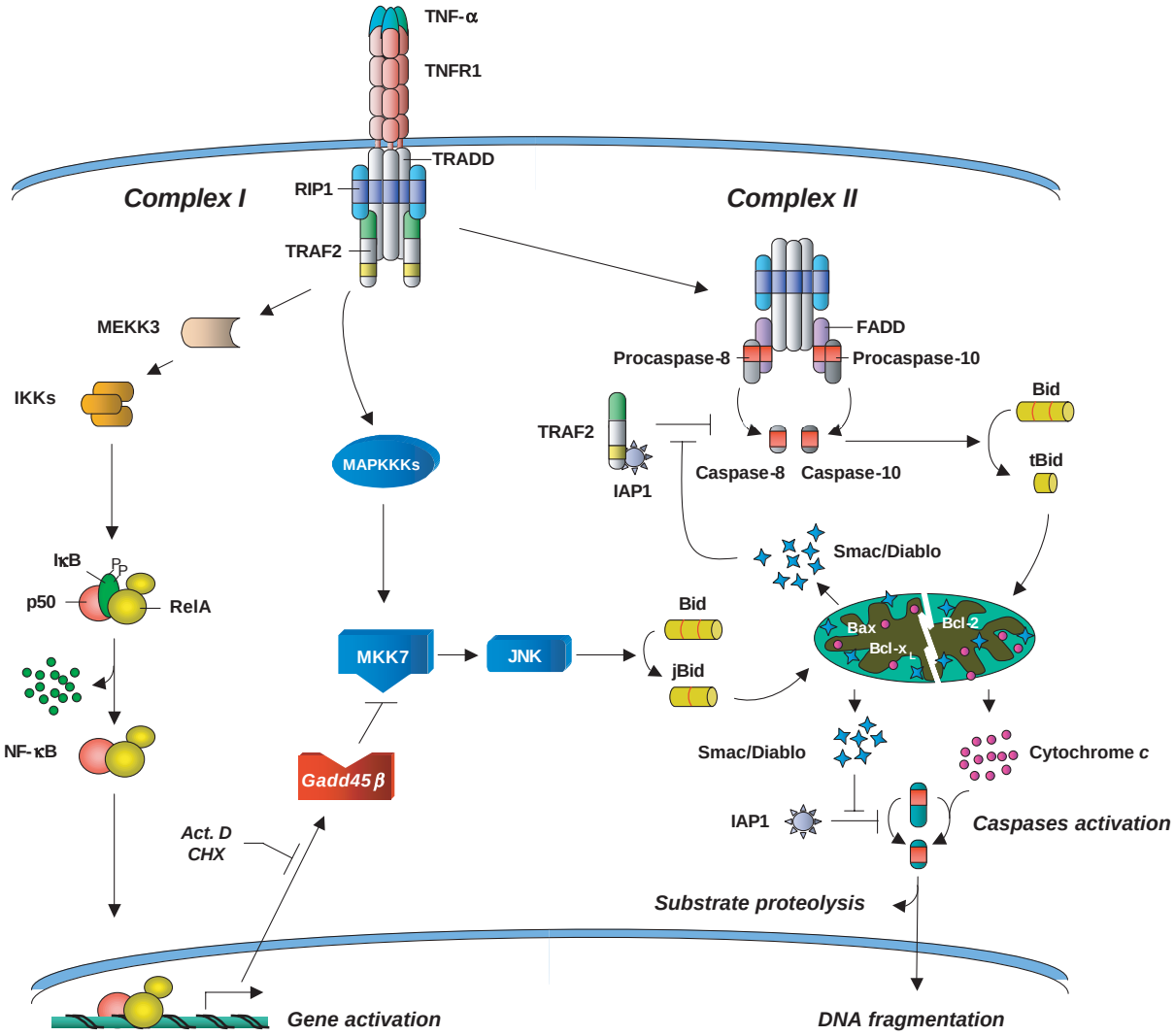

Fig. 1. TNFR1-induced pathways modulating apoptosis. Formation of complex I leads to NF-κB activation, Gadd45β induction, JNK inhibition and cell survival. Formation of complex II leads to caspase-8/10-mediated cleavage of Bid into tBid, which then targets mitochondria to induce cytochrome *c* release and, ultimately, cell death. The figure also depicts JNK activation, which results in formation of jBid; this promotes PCD by triggering release of Smac/Diablo into the cytosol, inhibiting the TRAF2-IAP1 complex and consequently activating caspase-8. The Gadd45β-MKK7 interaction linking the JNK and NF-κB pathways is also shown.

(Ghosh and Karin, 2002; Senftleben et al., 2001; Xiao et al., 2001).

Anti-apoptotic functions of NF-κB

Numerous studies have shown that NF-κB has anti-apoptotic effects that have been implicated in a variety of biological processes. In the B-cell lineage, this activity is required for completion of various developmental steps, including differentiation into mature IgM^{low}/IgD^{high} cells, as well as the response of these cells to antigen and CD40 costimulation (Gilmore et al., 2004; Gerondakis and Strasser, 2003). Likewise, during an immune reaction, survival of naive T cells depends on NF-κB activation by the T-cell receptor (TCR) and CD28 stimulation (Green, 2003; Zheng et al., 2003; Kane et al., 2002). NF-κB also plays an important pro-survival role during thymocyte development (Voll et al., 2000; Boothby et al., 1997; Esslinger et al., 1997).

The control of apoptosis by NF-κB is also crucial outside the immune system. Deletion of *RelA* in mice causes embryonic lethality at mid-gestation owing to massive liver apoptosis (Beg et al., 1995). A hepato-protective role of NF-κB in adults has now been confirmed by studies of animal models of TNF receptor (TNFR)-mediated damage (Chaisson et al., 2002; Maeda et al., 2003). Recently, the NF-κB pro-survival activity has also been implicated in epidermal homeostasis and hair follicle development, and accumulating evidence indicates that it might play an important role in the central nervous system (reviewed by Kucharczak et al., 2003; Mattson and Camandola, 2001).

NF-κB and cancer

The association between NF-κB and cancer dates as far back as the discovery of the molecule, when the viral oncoprotein v-Rel was identified as the causative agent of acute avian

leukemia (Gilmore, 1999). However, only recently has the extent to which NF-κB is involved in mammalian oncogenesis become apparent. Genes encoding NF-κB-family members such as p52/p100, Rel, RelA and the IκB-like protein Bcl-3 are frequently rearranged or amplified in human lymphomas and leukemias, and inactivating mutations of IκBα occur in Hodgkin's lymphoma (HL) (reviewed by Kucharczak et al., 2003; Orlowski and Baldwin, 2002; Karin et al., 2002). Moreover, virtually all oncogene products, including the Tax protein of human T-cell leukemia virus type 1 (HTLV-I), EBNA2 and LMP-1 of Epstein–Barr virus (EBV), Bcr-Abl, Her-2/Neu and oncogenic variants of Ras, can induce NF-κB (Kucharczak et al., 2003; Orlowski and Baldwin, 2002), and targeted overexpression of Rel in the mammary epithelium causes tumors (Romieu-Mourez et al., 2003).

Direct evidence from both in vivo and in vitro models indicates that control of apoptosis by NF-κB is crucial to its promotion of oncogenesis (Karin et al., 2002; Orlowski and Baldwin, 2002; Kucharzack et al., 2003). In the early stages of tumorigenesis, NF-κB suppresses transformation-associated apoptosis induced by oncoproteins such as mutated H-Ras and Bcr-Abl (Kucharzack et al., 2003; Orlowski and Baldwin, 2002). It is also needed for survival of a growing list of late-stage tumors, including HL, diffuse large B-cell lymphoma (DLBCL), multiple myeloma (MM), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML) and breast cancer (reviewed by Karin et al., 2002; Kucharzack et al., 2003; Orlowski and Baldwin, 2002). Both primary cancer tissues and cell culture models of these cancers exhibit constitutively active NF-κB, and inhibition of this activity by various means induces apoptosis.

Elevated NF-κB activity has also been associated with tumor resistance to anticancer therapy, as well as to TNF-α-induced apoptosis, which might help these cells evade immune surveillance (Greten and Karin, 2004; Kucharzack et al., 2003; Orlowski and Baldwin, 2002). Notably, NF-κB induction by chemotherapeutic agents can blunt their efficacy even when NF-κB is not constitutively active (Kucharzack et al., 2003; Orlowski and Baldwin, 2002). Indeed, blockers of NF-κB such as proteasome inhibitors (PS-341) are now being used successfully to treat patients with MM, and early clinical trials with these inhibitors are showing potential benefits against lymphoma, as well as prostate and lung cancer (Lenz, 2003; Richardson, 2003). Glucorticoids, which also block NF-κB, are part of the therapeutic regimen for HL.

Viral pathogenesis

The pro-survival activity of NF-κB also plays a crucial role in viral pathogenesis (reviewed by Kucharzack et al., 2003). Indeed, the need for an inducible gene expression program to maintain cell survival might have originally evolved as a mechanism for disposing of infected cells that, because of viral takeover, exhibit grossly defective transcription. Not surprisingly, many viruses have adapted to this host defense mechanism by developing their own anti-apoptotic strategies or acquiring genes that either induce or mimic NF-κB. Examples of such genes, many of which are implicated in viral oncogenesis, include: v-FLIP of human herpesvirus 8 (HHV-8), which is linked to Kaposi's sarcoma and lymphoma; Tax of HTLV-1, which causes adult T-cell leukemia (ATL); and of course v-Rel, which is encoded by the avian retrovirus REV-T (Kucharczak et al., 2003; Benedict et al., 2002).

The NF-κB-mediated control of PCD induced by TNFRs

The paradigm of the anti-apoptotic activity of NF-κB is the control of PCD induced through TNFRs is central to the anti-apoptotic activity of NF-κB. Indeed, it was studies of the biological responses to these receptors that originally revealed this function (Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996; Beg and Baltimore, 1996). The effects were later found to extend to other 'death receptors' (DRs), including Fas/CD95 and, probably, TRAIL-R1/DR4 and TRAIL-R2/DR5 (Kucharczak et al., 2003). TNF-α is a pleiotropic cytokine that plays a key role in inflammation, immunity, apoptosis and differentiation, and is arguably the most potent inducer of NF-κB (Aggarwal, 2003; Wajant et al., 2003). Signaling through its receptors can trigger PCD (Aggarwal, 2003; Wajant et al., 2003). Indeed, during a physiological immune response, TNFRs (and Fas) are crucial mediators of activation-induced cell death (AICD) of antigen-specific T cells (Baumann et al., 2002; Singer and Abbas, 1994; Sytwu et al., 1996; Zheng et al., 1995). They also participate in neuronal death after ischemic brain injury (Martin-Villalba et al., 2001) and cisplatinum-induced nephrotoxicity in mice (Ramesh and Reeves, 2002). Moreover, TNF-α exhibits cytotoxic activity in some tumor cell lines in vitro (Sugarman et al., 1985).

Normally, however, stimulation with the cytokine has no apoptotic effect unless NF-κB activation or protein synthesis is blocked (Kucharczak et al., 2003). Interestingly, these conditions can occur naturally. During infection with human adenovirus, virally encoded E1A suppresses NF-κB and sensitizes cells to TNF-α-induced death (Perez and White, 2003; Shao et al., 1997; Shao et al., 1999). Likewise, reovirus-induced neuronal apoptosis depends on TNFRs and other DRs (Richardson-Burns et al., 2002), and the increased sensitivity of T cells from aged individuals to TNF-α-induced PCD has been associated with reduced activation of NF-κB (Aggarwal et al., 1999; Aggarwal et al., 2000; Gupta, 2002). Finally, NF-κB activation can be severely impaired in certain genetic diseases, such as incontinentia pigmenti – a rare disorder caused by inactivating rearrangements of the *IKKγ* gene (also known as *NEMO*) that sensitize cells to TNF-α-induced apoptosis (Smahi et al., 2002).

Tchopp and colleagues recently provided important insights into the bases for the dichotomy of TNF-α signaling (Micheau and Tschopp, 2003) (see also Wajant et al., 2003). Upon ligand engagement, TNFR1 recruits to its cytoplasmic tail the death domain (DD)-containing proteins TRADD and RIP1, which then form one of two complexes. Complex I – which also contains TRAF2 – binds to the TNFR1 tail, inducing NF-κB activation and, thereby, cell survival. When ubiquitylated, the TRADD-RIP1 complex instead localizes to the cytosol, where it associates with FADD, yielding complex II, which then recruits and activates procaspase-8 and procaspase-10 to induce cell death.

The suppression of TNF-α-induced apoptosis by NF-κB is crucial for the survival of the organism and its response to injury. In mice lacking RelA, liver apoptosis and embryonic lethality are rescued by deletion of TNFR1 (Alcamo et al.,

2001; Doi et al., 1999). The resistance to TNF-α-induced apoptosis that NF-κB confers on the liver has also been observed in adults (Chaisson et al., 2002; Maeda et al., 2003). Overactivation of NF-κB by TNF-α can be detrimental too. For instance, when caused by loss of the de-ubiquitinase CYLD, it inappropriately blocks apoptosis, thereby promoting oncogenesis (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). NF-κB-mediated inhibition of TNFR-induced PCD is also involved in chronic inflammatory diseases (Liu and Pope, 2003) (see below).

The pro-survival mechanisms of NF-κB-mediated protection

Transcriptional control

Although other modes of action might exist (see Kurcharczak et al., 2003), the suppression of apoptosis by NF-κB is, by and large, a transcriptional event. NF-κB-regulated genes that are capable of blocking PCD have been identified (reviewed by Karin and Lin, 2002; Kurcharczak et al., 2003). Interestingly, the NF-κB-activated pro-survival program appears to be specifically tailored for each tissue and biological context. The bases for this plasticity are not fully understood, but the program seems to be dictated by the particular milieu of NF-κB dimers and transcription factors present in each tissue, and the specific network of interactions and modifications induced by the apoptotic stimulus (Hoffmann et al., 2003).

In some circumstances, the genes that are most relevant to the NF-κB anti-apoptotic activity seem to have been identified. For instance, several studies now indicate that, in peripheral B and T lymphocytes, NF-κB pro-survival signaling induced by antigen receptor and CD40 or CD28 costimulation targets members of the Bcl-2 family such as Bcl-x_L, Bfl-1/A1 and Bcl-2 itself (Grossmann et al., 2000; Grumont et al., 1999; Hsu et al., 2002; Khoshnan et al., 2000; Lee et al., 1999; Willis et al., 2003; Wu et al., 1996; Zong et al., 1999). However, in many other instances, including oncogenesis and cancer chemoresistance, the critical targets of NF-κB remain largely unknown.

The resistance to TNF-α-induced apoptosis that NF-κB confers has been associated with: upregulation of: the Bcl-2 family members Bcl-x_L and A1/Bfl-1; the catalytically inactive relative of caspase-8, $FLIP_L$; the combination of TRAF1/2 and inhibitor of apoptosis protein (IAP) 1/2; and X-chromosome-linked IAP (XIAP; also known as hILP) (reviewed by Karin and Lin, 2002; Kurcharczak et al., 2003). NF-κB might also induce expression of the cathepsin inhibitor, Spi2A, to blunt the lysosomal pathway for PCD (Liu et al., 2003). Indeed, these factors appear to be important mediators of anti-apoptotic NF-κB signaling in certain tissues, and their effects on apoptotic pathways have been well characterized. Nevertheless, most of these factors are only expressed in certain cell types, and their levels are not controlled by NF-κB or TNF-α in others. Moreover, they can only partly inhibit PCD in NF-κB-deficient cells. Hence, they cannot fully account for the protective effects of NF-κB (see De Smaele et al., 2001).

Suppression of JNK signaling

Several groups have shown that there is a crosstalk between the NF-κB and JNK pathways (De Smaele et al., 2001; Tang et al., 2001; Javelaud and Besacon, 2001). The JNK isoforms (JNK1-JNK3) are the downstream components in one of the major mitogen-activated protein (MAP) kinase cascades (reviewed by Chang and Karin, 2001; Davis, 2000). Normally, signaling through the JNK and p38 MAP kinase cascades is associated with induction of cell death, whereas signaling through the ERK MAP kinase cascade promotes cell growth and survival.

The pro-apoptotic role of JNK is evident from analyses of JNK-knockout mice. Mouse embryonic fibroblasts (MEFs) lacking both JNK1 and JNK2 are resistant to PCD induced by various stress stimuli, and *JNK3*$^{-/-}$ neurons have severely impaired apoptotic responses to excitotoxins (Davis, 2000; Tournier et al., 2000; Yang et al., 1997). Moreover, knocking out either JNK1 or JNK2 can protect peripheral T cells against AICD, and JNK1-deficient or JNK2-deficient thymocytes are refractory to anti-CD3-induced death in vivo (Arbour et al., 2002; Sabapathy et al., 1999; Sabapathy et al., 2001) (see also Dong et al., 2002; Rincon and Pedraza-Alva, 2003). Consistent with this pro-apoptotic role of JNK is the observation that T cells lacking JNK1 or the JNK kinase MKK7/JNKK2 mount a hyper-proliferative response to antigen stimulation (Dong et al., 1998; Sasaki et al., 2001).

There is now general agreement that JNK also plays an important role in TNFR-mediated apoptosis. Normally, however, cells survive TNF-α treatment (see above) and, thus, JNK inhibition has no apparent effect on cell viability – this might explain why early studies failed to uncover a pro-apoptotic role for JNK in TNFR signaling (Franzoso et al., 2003). In fact, this role was revealed by analyses performed under conditions allowing PCD – that is, inhibition of NF-κB.

Inhibition of JNK signaling by pharmacological agents or dominant-negative kinase mutants effectively rescues NF-κB-deficient cells from TNF-α-induced death (De Smaele et al., 2001; Tang et al., 2001; Javelaud and Besacon, 2001). Likewise, knocking out MKK7/JNKK2 virtually abrogates TNF-α-induced death in RelA-null cells (Deng et al., 2003). Expression of IκBαM – a potent blocker of NF-κB – yields similar results in *JNK1*$^{-/-}$ and *JNK2*$^{-/-}$ MEFs (Lamb et al., 2003) (F.Z. and G.F., unpublished observations). Furthermore, fibroblasts lacking the TNFR-induced MAP kinase kinase kinase (MAPKKK) apoptosis signal-regulated kinase 1 (ASK1) – an upstream activator of JNK (and p38) (Davis, 2000) – are significantly protected against the apoptotic effects of the cytokine (Tobiume et al., 2001). Interestingly, however, like JNK-null MEFs, these cells retain normal sensitivity to Fas-induced killing (Tobiume et al., 2001; Tournier et al., 2000), which suggests that JNK does not participate in this killing. Nevertheless, this might still occur in certain tumor lines (Zazzeroni et al., 2003a).

The relevance of the JNK cascade to apoptosis signaling is highlighted by the finding that activation of this cascade is controlled by NF-κB. Indeed, suppression of NF-κB by ablation of RelA or IKKβ, or expression of IκBαM, leads to persistent (rather then transient) JNK induction by TNF-α, and it seems to be the persistence of this induction that ultimately causes the cell to succumb to PCD (De Smaele et al., 2001; Javelaud and Besancon, 2001; Tang et al., 2001) (see also Franzoso et al., 2003). Caspases can activate various MAPKKKs (Davis, 2000; Roulston et al., 1998), but the effects of NF-κB on JNK signaling are not affected by protective cell treatment with the caspase blocker z-VAD_{fmk} and so do not

appear to be a secondary consequence of caspase inhibition (Javelaud and Besancon, 2001; Franzoso et al., 2003). In short, the containment of the JNK cascade is crucial for the control of TNF-α-induced apoptosis, and this critically depends on NF-κB. Curiously, although confirming the inhibitory effects of NF-κB on JNK signaling, another study has suggested that, in TNF-α-treated NF-κB-deficient cells, persistent JNK activation promotes cell survival (Reuther-Madrid et al., 2002). The bases for the discrepancy with other studies are not clear.

Clearly, there are also JNK-independent mechanisms by which TNFRs can trigger PCD. In NF-κB-deficient cells, protection by JNK inactivation is not complete, and JNK-null MEFs eventually succumb to treatment with TNF-α (De Smaele et al., 2001; Javelaud and Besancon, 2001; Tang et al., 2001; Lamb et al., 2003). Nevertheless, there is now compelling evidence to demonstrate an obligatory role for JNK in efficient apoptosis in response to the cytokine. Paradoxically, in most cells, activation of JNK by TNF-α occurs without significant death. This is probably because – unlike UV and other stress stimuli – TNF-α only causes transient elevation of JNK activity, which normally is not a signal for PCD (Davis, 2000; Franzoso et al., 2003). For pro-apoptotic activity, JNK must signal chronically, as during inhibition of NF-κB. Indeed, constitutive JNK activation – through expression of MKK7-JNK fusion proteins – seems sufficient on its own to induce cell death (Lei et al., 2002). Still, the actual role of JNK in TNF-α-induced apoptosis signaling might be influenced by cell-type-specific elements.

The importance of this antagonistic crosstalk between NF-κB and JNK has recently been documented in animal models. Karin and colleagues have shown that NF-κB activation is required to antagonize hepato-toxicity induced by systemic challenge with lipopolysaccharide (LPS) or concanavalin A (ConA) – two agents that provoke liver damage through TNFR-induced cell death (Maeda et al., 2003). This study shows that conditional deletion of IKKβ results in markedly increased induction of JNK by these agents and that suppression of this induction by ablation of either *JNK1* or *JNK2* blunts TNFR-mediated hepatic injury (Maeda et al., 2003). It is plausible that the pro-survival activity of NF-κB in the fetal liver may also involve attenuation of pro-apoptotic JNK signaling.

An evolutionarily conserved crosstalk

Evidence for the importance of the NF-κB–JNK crosstalk also comes from its evolutionary conservation. In *Drosophila*, the duration of JNK induction in response to LPS is directly controlled by the NF-κB protein Relish (Park et al., 2004). Here, LPS signals through the Imd pathway, which is named after the insect ortholog of the TNFR pathway kinase RIP and controls immunity to Gram-negative bacteria through two main branches: the JNK and IKK/Relish cascades (Hoffmann and Reichhart, 2002; Silverman and Maniatis, 2001). These branches diverge downstream of the MAPKKK TAK1 to direct transcription of distinct subsets of genes in a temporally coordinated manner (Boutros et al., 2002). Whereas Relish-responsive genes exhibit sustained expression, JNK targets are characterized by transient induction, owing to Relish-mediated shut down of JNK signaling – a process involving proteosomal degradation of TAK1 promoted by unidentified Relish targets (Park et al., 2004). Notably, the Relish-mediated attenuation of the JNK cascade might also control apoptosis. Indeed, the *Drosophila* TNF-α homolog, Eiger, depends on JNK – rather than on the caspase-8 homolog, Dredd – to induce death (Igaki et al., 2002; Moreno et al., 2002). Thus, the role of JNK in apoptosis signaling appears to be a remnant of a primordial death mechanism engaged by TNF-α, which only later in evolution began to exploit the FADD/caspase-dependent pathway, yet maintaining its connection to NF-κB.

Mechanisms for TNFR-induced JNK pro-apoptotic signaling

Whereas in some circumstances, JNK-induced apoptosis involves modulation of gene expression, apoptosis induced by stress stimuli or TNF-α appears to be mediated by factors that are already present in the cell (Davis, 2000; Tournier et al., 2000). A recent study has shown that JNK activation by TNF-α causes caspase-8-independent processing of Bid, a BH3-only member of the Bcl-2 family (Bouillet and Strasser, 2002; Danial and Korsmeyer, 2004), into jBid, which then targets mitochondria to trigger selective release of the apoptogenic factor Smac/Diablo (Fig. 1) (Deng et al., 2003) (see also Chai et al., 2000; Verhagen et al., 2000). In the cytosol, this factor then binds to and antagonizes IAP1, thereby relieving caspase-8 from the inhibitory effects of the TRAF2-IAP1 complex (see also Wajant et al., 2003). In keeping with the evolutionary conservation of the JNK apoptotic mechanism in flies, Eiger-induced JNK activation in flies triggers death through Hid, Reaper and Grim, the functional equivalents of mammalian Smac/Diablo (Igaki et al., 2002; Moreno et al., 2002).

The JNK-jBid-Smac/Diablo pathway represents a newly identified link between the intrinsic (mitochondrial) and extrinsic (DR) pathways of apoptosis. In this link (seemingly specific for TNFRs), mitochondria appear to lie upstream of caspase-8 (Deng et al., 2003), which contrasts with the 'classical', caspase-8–tBid–cytochrome-*c* pathway (shared by Fas and TRAIL-Rs), in which mitochondria are downstream of this caspase (Fig. 1) (Barnhart et al., 2003). Interestingly, this might explain the somewhat puzzling observation that, despite greatly impaired JNK induction and near-normal NF-κB activity, TRAF2-null cells exhibit hypersensitivity to TNF-α-induced apoptosis (Yeh et al., 1997). According to this model, loss of TRAF2 should prevent recruitment of IAP1 to the TRADD–FADD–caspase-8 complex, thereby causing caspase-8 activation without a need for JNK-mediated release of Smac/Diablo (Fig. 1).

Why does only prolonged JNK induction lead to cell death? One possibility is that the effectors of JNK pro-apoptotic signaling become available in the cell only some time after TNF-α stimulation. Alternatively, these effectors (for instance, jBid or Smac/Diablo) might have to accumulate in significant amounts before they can trigger apoptosis. It is also possible that the JNK apoptotic activity can be counteracted, but only temporarily, by concomitant activation of pro-survival pathways, such as the ERK, Akt/PKB or perhaps NF-κB pathway (Chang and Karin, 2001; Davis, 2000). Indeed, despite the well-documented ability of JNK to trigger apoptosis, the actual biological response to its activation also depends (apart, of course, from its duration) upon the stimulus and tissue (Chang and Karin, 2001; Davis, 2000).

JNK and pro-survival signaling

Curiously, in the presence of NF-κB, transient JNK activation might actually protect cells from apoptosis following TNFR stimulation. Recent studies of *JNK1*$^{-/-}$ and *JNK2*$^{-/-}$ fibroblasts have shown that this is mediated by the transcription factor JunD, which might collaborate with NF-κB to upregulate expression of anti-apoptotic factors such as IAP2 (Lamb et al., 2003). Thus, in TNF-α-treated cells, NF-κB-mediated survival could require transient activation of JNK. Note, however, that these studies used nonspecific inhibitors of protein synthesis, such as CHX and emetine, and there is evidence that these inhibitors can bypass the requirement for JNK in death signaling (Deng et al., 2003).

Gadd45β and MKK7: critical targets

NF-κB controls the JNK cascade by inducing target genes. Curiously, these targets seem to differ between insects and mammals. Screens for cDNAs that block TNF-α-induced apoptosis in *RelA*$^{-/-}$ fibroblasts identified Gadd45β (also known as Myd118) – one of a family of structurally related, acidic and predominantly nuclear proteins – as an effector of the suppressive activity of NF-κB (De Smaele et al., 2001). Although their exact structures and functions are not known, Gadd45 proteins have been implicated in cell-cycle control, DNA repair and several other processes (Carrier et al., 1999; Nakayama et al., 1999; Vairapandi et al., 2000; Wang et al., 1999a; Yang et al., 2000; Zhan et al., 1999; Zhang et al., 1999; Zhao et al., 2000). Gadd45β upregulation by TNF-α requires NF-κB, and expression of Gadd45β in NF-κB-null cells abrogates induction of the JNK cascade by TNF-α. Importantly, this expression also blocks the caspase-independent phase of TNF-α-induced JNK signaling, and Gadd45β inactivation blunts the normal downmodulation of this signaling (De Smaele et al., 2001; Papa et al., 2004). Hence, Gadd45β is a bona fide inhibitor of the JNK pathway.

The JNK kinase MKK7/JNKK2 is a target of Gadd45β and, indirectly, of NF-κB (Papa et al., 2004). Gadd45β associates tightly with MKK7 and inhibits its catalytic activity by contacting crucial residues in the catalytic pocket, including the ATP-binding residue Lys149 (Moriguchi et al., 1997). It probably inactivates MKK7 by blocking access of ATP (Papa et al., 2004). MKK7 is a selective activator of JNK, and its ablation in fibroblasts completely abolishes JNK induction by TNF-α (Davis, 2000; Tournier et al., 2001). Thus, blocking this MAPKK seems sufficient alone to account for the specific and near-complete inhibition of the JNK cascade by Gadd45β (De Smaele et al., 2001; Papa et al., 2004). Studies using peptides that impede binding of Gadd45β to MKK7 support this notion and show that, at least in some tissues, the Gadd45β-mediated targeting of MKK7 is crucial for the protective effects of NF-κB (Papa et al., 2004). Thus, the Gadd45β-MKK7 interaction represents an important molecular link between the NF-κB and JNK pathways (Fig. 1).

In contrast with these findings, a recent report has suggested that, in MEFs, Gadd45β ablation has no effect on TNF-α-induced PCD (Amanullah et al., 2003). Whereas this discrepancy might in part reflect experimental differences (Zazzeroni et al., 2003b), there could be other explanations. The mechanism of MKK7 inhibition activated in response to TNF-α appears to be tissue specific and, at least in MEFs, other factors (distinct from Gadd45β) contribute to block induction of this JNK kinase (Papa et al., 2004). Yet, despite the presence of these factors, experiments with cell-permeable peptides show that, in these cells, Gadd45β is required for efficient blocking of TNF-α-induced killing (Papa et al., 2004).

In some systems, overexpression of Gadd45 factors has been linked to apoptosis (Chung et al., 2003; Takekawa and Saito, 1998; Vairapandi et al., 2000). It is not clear, however, whether this is physiological, because in many other systems induction of endogenous Gadd45 polypeptides is associated with cell survival, and overexpression of these polypeptides triggers no apparent toxicity (Yang et al., 2001; De Smaele et al., 2001; Nakayama et al., 1999; Smith et al., 1996; Smith et al., 2000; Hoffmeyer et al., 2001; Zazzeroni et al., 2003a). These discrepancies probably reflect tissue-specific effects of these polypeptides and/or distinct activities of the different family members: Gadd45α (Gadd45), Gadd45β and Gadd45γ (OIG37/CR6). For instance, Gadd45β is unable to inhibit cell growth in many systems (De Smaele et al., 2001; Yang et al., 2000; Yang et al., 2001; Zazzeroni et al., 2003a) and, whereas various tumor cell lines and primary cells tolerate high levels of Gadd45β, they cannot sustain stable overexpression of Gadd45α (De Smaele et al., 2001; Zazzeroni et al., 2003a; Yang et al., 2000; Yang et al., 2001) (S.P., F.Z. and G.F., unpublished observations).

Gadd45 proteins and other aspects of MAPK signaling

Numerous studies have now implicated Gadd45 proteins in the regulation of MAPK signaling at several levels. Gadd45 polypeptides associate not only with MKK7, but also with the MAPKKK MEKK4/MTK1 (Takekawa and Saito, 1998; Mita et al., 2002). In the latter case, they might serve as initiators of p38 and JNK signaling in response to stress (Takekawa and Saito, 1998; Mita et al., 2002). In keratinocytes, Gadd45α is seemingly involved in p38 and JNK signaling in response to UV irradiation (Hildesheim et al., 2002). The Gadd45β- and Gadd45γ-mediated control of MAPK cascades also appears to be essential for the differentiation/function of the T helper 1 (Th1) subset of T cells (Yang et al., 2001; Lu et al., 2001; Lu et al., 2004). Furthermore, knockout studies have placed these factors upstream of MEKK4 in the TCR- and interleukin (IL)-12 receptor-induced pathways for p38 activation (Chi et al., 2004).

The basis for the regulation of MAPK signaling by Gadd45 proteins and its function remain, however, somewhat controversial. Whereas *Gadd45β*$^{-/-}$ and *Gadd45γ*$^{-/-}$ Th1 cells exhibit a severe defect in p38 and JNK activation by both antigen and cytokine receptors (Lu et al., 2001; Lu et al., 2004), the signaling impairment in MEKK4-deficient lymphocytes is confined to the p38 cascade (Chi et al., 2004). This suggests that, in these cells, regulation of JNK signaling by Gadd45 involves a MEKK4-independent mechanism. Furthermore, the seemingly global signaling defect in Gadd45β-null and Gadd45γ-null T cells might be due, at least in part, to a developmental block – which would explain the unexpected loss of ERK activation, an event that is not believed to involve MEKK4 or to be controlled by Gadd45 protein (Davis, 2000; Takekawa and Saito, 1998).

Other observations complicate things further. Two studies

have shown that activation of JNK and p38 by stress precedes, rather than follows, induction of *Gadd45* genes (Shaulian and Karin, 1999; Wang et al., 1999b). Moreover, in several cell lines, overexpression of Gadd45 polypeptides does not cause JNK or p38 activation (Wang et al., 1999b; De Smaele et al., 2001), and a recent study has shown that activation of p38 by Gadd45α involves direct association with this MAPK rather than with MEKK4 (Bulavin et al., 2003). In addition to MEKK4, Gadd45β interacts with the MAPKKK ASK1 (Papa et al., 2004). Nevertheless, association with these two MAPKKKs does not appear to be relevant to the Gadd45β-mediated control of JNK activation and PCD induced by TNF-α, because MEKK4 is not involved in TNFR signaling (Davis, 2000; Takekawa et al., 1997), and ASK1 is seemingly unaffected by Gadd45β (Papa et al., 2004). Furthermore, activation of p38 (possibly through interaction of Gadd45β with MEKK4 or ASK1) would be unlikely to modulate the apoptotic response to TNF-α (De Smaele et al., 2001), and any activation of JNK through a similar mechanism would be overridden by downstream suppression of MKK7 (Papa et al., 2004).

These apparent discrepancies probably reflect the complexity of the biological functions of Gadd45 factors, which are probably subject to tissue- and/or signal-specific regulation that ultimately dictates their output.

Other mechanisms of NF-κB–JNK crosstalk

Additional factors might mediate inhibitory effects of NF-κB on JNK signaling (Fig. 2). One such factor is XIAP, a member of the IAP family of caspase blockers (Clem, 2001; Salvesen and Duckett, 2002). XIAP binds to and inhibits caspase-3 and caspase-7 through its N-terminal linker and baculovirus IAP repeat (BIR) 2 domain, respectively, and might prevent activation of procaspase-9 through a BIR3-containing region (Takahashi et al., 1998; Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001) (see also Clem, 2001; Karin and Lin, 2002). It is also a known target of NF-κB (Stehlik et al., 1998), and its induction by TNF-α is somewhat reduced in *RelA*$^{-/-}$ MEFs (Tang et al., 2001). Overexpression of XIAP inhibits TNF-α-induced cytotoxicity in NF-κB-deficient cells (Stehlik et al., 1998), and thymocytes from XIAP-transgenic mice are resistant to apoptosis induced by various triggers (Conte et al., 2001). This overexpression has been reported to diminish activation of JNK by TNF-α in *RelA*$^{-/-}$ cells, but to have no effect on the p38 and ERK cascades (Tang et al., 2001). However, the mechanisms by which XIAP blocks this activation are not clear. One possibility is that this is a secondary consequence of caspase inhibition (Salvesen and Duckett, 2002). Nevertheless, like Gadd45β, XIAP can block both the caspase-dependent phase and caspase-independent (that is, z-VAD$_{fmk}$-insensitive) phase of JNK induction by TNF-α (also discussed above) and thus may be a genuine inhibitor of the JNK cascade. Indeed, XIAP is capable of interacting with JNK-activating kinases (Yamaguchi et al., 1999; Sanna et al., 2002). Curiously, however, these interactions appear to result in activation rather than inhibition of JNK signaling (Sanna et al., 2002). Furthermore, *XIAP*$^{-/-}$ mice exhibit no obvious apoptotic phenotype (Harlin et al., 2001), and XIAP ablation in MEFs does not affect the kinetics of JNK induction by TNF-α (Kucharczak et al., 2003). Thus, although this ablation might activate compensatory mechanisms, further studies are needed to establish whether XIAP-mediated control of JNK is physiological.

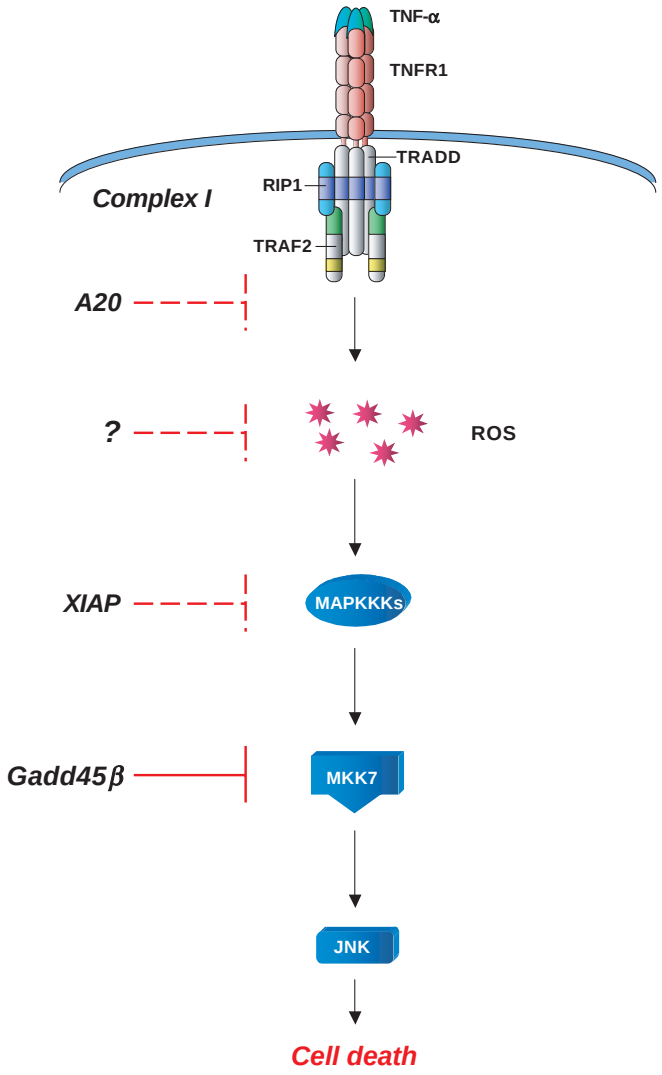

Fig. 2. The various mediators of the NF-κB suppression of JNK signaling and their potential modes of action.

Another likely candidate is the zinc-finger protein A20. It is rapidly induced by TNF-α through a mechanism that requires NF-κB (Beg and Baltimore, 1996; Kucharczak et al., 2003). A20-null MEFs exhibit increased apoptosis after treatment with TNF-α, and this correlates with increased activation of JNK (Lee et al., 2000) (see also Lademann et al., 2001). However, overexpressed A20 cannot block PCD in NF-κB-deficient cells (Beg and Baltimore, 1996). Thus, although required, it does not appear to be sufficient to mediate the pro-survival activity of NF-κB. How A20 blunts JNK signaling is not understood. Nevertheless, A20 can interact with TRAF2 and NEMO and is recruited to the TNFR1-IKK signaling complex upon stimulation with TNF-α (Song et al., 1996; Zhang et al., 2000), which suggests that it acts immediately downstream of the receptor (Lee et al., 2000; Lademann et al., 2001; Song et al., 1996; He and Ting, 2002). Its upregulation

also blocks TNF-α-induced NF-κB activation and, in fact, is an important negative-feedback mechanism controlling this (Lee et al., 2000; Song et al., 1996) (see also Wajant et al., 2003). Analyses of *A20*$^{-/-}$ MEFs suggest that A20 specifically interferes with activation of NF-κB and JNK signaling in response to TNF-α, because induction of this signaling by IL-1 is unaffected in these cells (Lee et al., 2000). This is in line with an early report proposing that NF-κB acts selectively on JNK activated by TNFR signaling and not by IL-1R or UV rays (Tang et al., 2001). Note, however, that a more recent study has shown that NF-κB negatively regulates JNK activation regardless of whether the cell stimulus is TNF-α or IL-1 (Reuther-Madrid et al., 2002), and various biochemical studies support a more global role for A20 in regulating MAPKKK activity and cytokine receptor signaling (Zetoune et al., 2001; Heyninck et al., 1999; Jaattela et al., 1996). Thus, at the present time, it is not possible to propose a comprehensive, straightforward model depicting the function of A20.

An intriguing new study has shown that NF-κB activation prevents accumulation of reactive oxygen species (ROS) produced in response to TNF-α – probably through upregulation of target genes (Sakon et al., 2003) (C.G.P. and G.F., unpublished observations). This is relevant since ROS have been implicated as essential mediators of TNF-α-induced apoptosis (Wajant et al., 2003) (see also Sakon et al., 2003; Schulze-Osthoff et al., 1993). Expression of the superoxide dismutase Mn-SOD – a ROS scavenger and known target of NF-κB (Bernard et al., 2001; Tanaka et al., 2002; Wong et al., 1989) – cannot reproduce the effects of NF-κB on ROS generated in response to TNF-α (Sakon et al., 2003). This indicates that additional factors are responsible. Because ROS help to sustain JNK signaling – partly through inactivation of the ASK1 inhibitor thioredoxin (Matsuzawa et al., 2002) – their suppression might represent yet another mechanism by which NF-κB downregulates this signaling.

NF-κB–JNK crosstalk and human disease

TNF-α is a potent activator of NF-κB, which in turn is a potent inducer of TNF-α (Aggarwal, 2003). This positive feedback is key to chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (Tak and Firestein, 2001; Romas et al., 2002). Indeed, the standard therapy for these conditions includes NF-κB blockers such as aspirin and glucocorticoids, and neutralizing anti-TNF-α antibodies represent an effective new tool (Makarov, 2000; Roshak et al., 2002). Inhibition of NF-κB by either glucocorticoids or proteasome inhibitors is also beneficial in certain malignancies, including HL and MM (Tesch et al., 2001; Lenz, 2003; Richardson, 2003). However, current compounds can only achieve partial inhibition of NF-κB and have considerable side effects, which limit their use in humans. Thus, there is an urgent need for new drugs that target the downstream anti-apoptotic effectors rather than NF-κB itself.

The finding that the targeting of JNK signaling by NF-κB is a key protective mechanism mediated by NF-κB offers a unique opportunity for developing such drugs. Blocking the ability of NF-κB to shut down JNK activation should promote apoptosis of self-reactive and proinflammatory cells at the site of inflammation, where there are high levels of TNF-α (Tak and Firestein, 2001; Romas et al., 2002). Experiments using cell-permeable peptides indicate that compounds that disrupt binding of Gadd45β to MKK7 might provide an effective new tool (Papa et al., 2004) (Fig. 3). These compounds or others that interfere with the NF-κB–JNK crosstalk might enable us to dissociate the anti-apoptotic and proinflammatory functions of NF-κB and so avoid the potent immunosuppressive effects of global NF-κB blockers (Karin et al., 2004; Tak and Firestein, 2001). Indeed, given the apparent cell-type specificity of the JNK inhibition program activated by NF-κB (Papa et al., 2004), they might also allow selective targeting of this program in diseased tissues.

This therapeutic relevance might also extend to cancer (Franzoso et al., 2003). JNK and NF-κB have seemingly opposing effects in tumor cells. Whereas NF-κB activation is required to suppress transformation-associated apoptosis (Kucharczak et al., 2003), activators of the JNK cascade (e.g. MKK4, JNK3 and BRCA1) are tumor suppressors (Franzoso et al., 2003; Kennedy and Davis, 2003). Several oncogene products, including oncogenic Ras and Her-2/Neu, are potent inducers of JNK (Davis, 2000; Kennedy and Davis, 2003), and thus cancerous cells might need constitutively active NF-κB to suppress JNK-mediated apoptosis induced by these products. Apoptosis caused by genotoxic agents such as topoisomerase inhibitors – a desirable effect in anticancer therapy – also requires JNK (Davis, 2000; Tournier et al., 2000) but is antagonized by NF-κB (Kucharczak et al., 2003). Thus, augmentation of JNK signaling by inhibiting selected NF-κB targets might provide a powerful new adjuvant for cancer treatment.

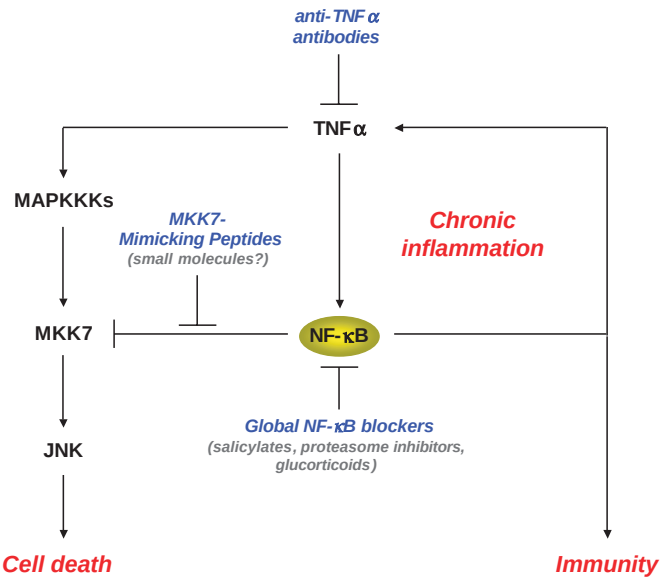

Fig. 3. Potential therapeutic implications of the NF-κB-mediated blockade of TNF-α-induced JNK signaling. A positive-feedback loop between TNF-α and NF-κB drives chronic inflammatory states; several pharmacological agents can be used to treat these states. The effects of MKK7-mimicking peptides are also shown (Papa et al., 2004), which support the therapeutic feasibility of blocking the NF-κB anti-apoptotic activity without significantly affecting the immune system.

Conclusions

The NF-κB-mediated attenuation of JNK signaling is crucial for numerous physiological processes, such as the response of the liver to injury and the survival of cells during an inflammatory reaction, as well as for chronic inflammatory diseases and cancers. In recent years, much progress has been made in understanding the basis for this attenuation, and it is now clear that this involves activation of a program of gene expression. The use of multiple genes might ensure effective shut down of the JNK cascade. It might also enable the organism to tailor the response to specific biological contexts and needs. Undoubtedly, a major future challenge will be to determine which NF-κB-inducible genes are most crucial in each context and, ultimately, how their products inhibit JNK signaling. Analyses of conditional knockout models will be invaluable to address these issues. Since inappropriate NF-κB-mediated blockade of apoptosis is key to several human diseases, these efforts could lead to the development of new treatment strategies. Initial findings suggest that it might be possible to achieve tissue-specific inhibition of the NF-κB anti-apoptotic activity with minimal side effects on the immune system. Indeed, this represents a major therapeutic goal.

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